Evaluation of Microbiome on Chicken Necrotic Enteritis and Growth Performance

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Evaluation of Microbiome on Chicken Necrotic Enteritis and Growth Performance

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Poultry Science

by

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ABSTRACT

Necrotic enteritis (NE) has re-emerged following restriction of antimicrobial usage and costs $6 billion every year worldwide. The primary objective of the studies was to evaluate prevention and treatment of NE using a microbiota metabolic product, secondary bile acid deoxycholic acid (DCA), in drinking water. Day-old birds were tagged and placed in floor pens. In experiments 1 and 2, the birds were infected with *Eimeria maxima* (*Em*) at d 18 and *C. perfringens* at d 23 and 24 and the birds were euthanized at d 26. In experiment 3, birds were infected with *Eimeria* at d 20 and *C. perfringens* at d 25 and 26 and euthanized at d 27. DCA was administered in drinking water at 1.91, 0.95, or 0.48 mM, respectively. Small intestinal content and tissue were collected for histopathology and molecular study. Data were analyzed using one-way ANOVA followed by t-test. NE birds in experiment 1 developed subclinical signs of NE, while NE birds in experiment 3 suffered acute NE. DCA at 1.91 mM attenuated the BWG loss in experiments 1 and 3. Consistently, it reduced NE-induced intestinal histopathology in experiments 1 and 3. However, in experiment 2 because of minimal body weight alteration in *Em* and NE birds compared to non-infected birds, no tissue was collected. DCA reduced NE-induced expression of inflammatory gene *Infγ* in experiment 1. Similarly, DCA reduced elevation of NE-associated *C. perfringens* luminal colonization in experiment 1. Using targeted metabolomics (LC-MS/MS) of bile acids, we found that NE was associated with reduction of total bile acids in ileal content.

The second objective of the studies was to examine improvement of broiler chickens growth through modulating gut microbiota. Mouse specific pathogen free (SPF) stool was cultured on Brain Heart Infusion (BHI) agar under anaerobic or aerobic condition and collected as SPF anaerobic (SPF-Anaero) and aerobic (SPF-Aero) microbiota. Day-old birds were tagged, weighed and randomly assigned to 9 pens. The birds were then orally gavaged with PBS, $10^8$ CFU/bird.
SPF-Anaero, or $10^8$ CFU/bird SPF-Aero. Body weight was measured at d 0, 14, and d 28, and euthanized at d 28. Data was analyzed using one-way ANOVA followed by $t$-test. Birds colonized with SPF-Anaero grew 15% heavier during d 0-14 (28 vs. 24 g/day/bird, $P= 0.0009$) compared to control birds, while birds colonized with SPF-Aero gained 8% more body weight compared to control birds (26 vs. 24 g/day/bird, $P= 0.02$). Consistently, SPF-Anaero birds grew faster compared to control birds during d14-28 (67 vs. 60 g/day/bird, $P= 0.007$). SPF-Anaero and SPF-Aero birds grew 15 and 4%, respectively, heavier on accumulative body weight gain compared to control birds (47 vs. 42 g/day/bird, $P= 0.0009$; 44 vs. 42 g/day/bird, $P= 0.03$, respectively).

In conclusion, microbial metabolic product DCA in drinking water prevents NE-induced BWG reduction and histopathology, perhaps through reducing $C. perfringens$ colonization and/or bile acid restoration. Modulating microbiota could be used as an antimicrobial free alternative to improve bird body weight gain. These findings may offer an alternative strategy to use of DCA and microbiota for NE prevention and growth promotion.
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DEDICATION

I would like to dedicate this work to my family and friends who inspired and supported me throughout my life.
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Chapter I:

Literature Review
1.1. Introduction

Before the current name, necrotic enteritis (NE) caused by *C. perfringens* was first called *Bacillus welchii*, then renamed to *Clostridium welchii*, from intestinal lesions of Black Orpington pullets in 1930 by Bennetts, in Australia (Bennetts, 1930). Later on, the disease was induced by feeding *C. perfringens* culture to chickens and called the "six-day disease," when the bacteria were observed to infect intestinal mucosa of the birds (Johansson & Sarles, 1948). However, the term necrotic enteritis was coined and reported by Parish in 1961 in England (Parish, 1961). After the discovery of NE, methods had been developed to control the disease. Antibiotics were first used for NE prevention and treatment, and later on they are used as growth promoters. Antibiotic growth promoters (AGPs) have been widely used in food animal production for decades (Stokstad & Jukes, 1950). For example, in 1951, the United States Food and Drug Administration permitted the use of antibiotics in animal feed (F. T. Jones & Ricke, 2003). Before AGPs were banned by the European Union (EU) in 2006, antibacterial drugs, such as avoparcin, lincomycin, amoxicillin, tylosin virginiamycin, and bacitracin, were commonly used for both prevention and treatment of NE (Craven et al., 2001; McDevitt et al., 2006a). However, after observing the effects of AGPs on the development and expansion of drug-resistant bacteria, people started to understand its potential threat. Even though the primary threat was the dramatic increase in the emergence of bacterial antibiotic resistance, the effect of the drug withdrawal was not negligible (Fey et al., 2000; Hershberger et al., 2005). In poultry industry, the removal of AGPs from some commercial farms increased disease incidences, especially those economically important diseases such as *C. perfringens* induced NE and other enteric infections (Kumar et al., 2018). In Europe, studies show that, with the removal of AGPs, disease incidence has dramatically increased. As a result, antibiotics used in animal production as a treatment were elevated (Casewell, et al., 2003).
Although strict biosafety practices without AGPs can maintain production in some farms (Engster et al., 2002), it is difficult to rely on this approach for every farms. Therefore, AGPs alternatives are much needed to control NE and other diseases.

Necrotic enteritis is caused by *C. perfringens*, which is a gram-positive, spore-forming, and non-motile bacteria. *C. perfringens* is a normal inhabitant in the environment and in a healthy bird's intestine (McDonel et al., 1980), and it is one of the fastest-growing pathogenic bacteria (Shimizu et al., 2002). NE mostly occurs in broiler chicks between 2-6 weeks of age (Barnes et al., 1972).

Since *C. perfringens* cannot synthesize several essential amino acids, it releases enzymes that degrade host tissue to meet its demand (Shimizu et al., 2002). In humans and animals, the ability of *C. perfringens* to release toxins, to grow very fast in a wide range of temperatures, and to form spores enable them to induce disease. *C. perfringens* induces NE by adhering to the small intestine villi, multiplying and releasing toxins, which causes necrosis (Al-Sheikhly et al., 1977). Toxins released by *C. perfringens* play a critical role in the incidence of NE. So far, more than 17 different types of *C. perfringens* toxins have been studied (Losada-Eaton et al., 2008). Based on the types of toxins released, *C. perfringens* is categorized into five types (A- E) (McDonel, 1980), such as alpha (α), beta (β), epsilon (ε) and iota (ι) (Miclard et al., 2009). Out of all types of toxins, alpha toxin is the only main toxin produced by all types of *C. perfringens*. Genes of *C. perfringens* toxins can be found in both chromosomes and plasmids. The alpha-toxin is considered the first known enzyme and main fatal bacterial toxin (Naylor et al., 1999). The alpha-toxin is an enzyme, phospholipase C sphingomyelinase that damages the phospholipids of the cell membrane. Thus, the alpha-toxin leads to the destruction of the cell membrane and the cell death by hemolysis or necrosis (Naylor et al., 1999). Even though the alpha-toxin is responsible for myonecrosis of gas gangrene, its effect on NE is controversial (Awad et al. 1995). *C. perfringens* types A and C have
been the primary causative agents of NE. In type A strains, the alpha-toxin has phospholipase C activity and plays a vital role in the pathogenesis of *C. perfringens*. Al-sheikhly et al. (1977) induced NE by intro-duodenal infusions of *C. perfringens* crude toxins and broth culture into the chickens' gut. Based on their findings, the alpha-toxin was the causative agent of NE (Al-sheikhly et al., 1977). Infected chickens had shown higher alpha-toxin levels compared to uninfected chickens (Hofshagen & Stenwig, 1992). Inoculation of germ-free chickens with either a broth culture supernatant or a purified alpha-toxin of *C. perfringens* type A induced mortality and intestinal lesions, whereas chickens challenged with an alpha-toxin and neutralized with an anti-alpha-toxin serum caused no deaths (Fukata et al., 1988). Birds with an NE history have shown a significant level of alpha-toxin antibodies (Lovland et al. 2003). Though *C. perfringens* type A strains are a normal inhabitant of the environment and healthy chicken gut, virulent strains produce higher levels of alpha-toxin. Rehman et al. (2009) in their *in vitro* study determined that intestinal mucosal barrier function was impaired by alpha-toxin (Rehman et al., 2009). However, since these studies were based on crude or partially-purified toxins, the toxins can still have other co-purifying proteins (Keyburn et al., 2006). Cooper & Songer (2009) have shown that creating NE lesions was independent of the amount of *in vitro* alpha-toxin produced, even though immunization with recombinant alpha-toxin provided partial protection against *C. perfringens* induced NE (Cooper & Songer, 2009). Another *in vitro* study using *C. perfringens* isolated from healthy and NE chickens showed no difference in alpha-toxin production (Gholami et al., 2006). Therefore, it remains uncertain whether alpha-toxin is the main virulence factor of NE.

The pore-forming toxin NetB was thought an indispensable virulence factor in NE pathogenesis (Keyburn et al., 2008). Keyburn et al. (2008) showed that NetB, not alpha-toxin, was responsible for the incidence of NE. They found similar NE between the wild type strain and alpha-toxin
negative mutants isolated from NE birds. In addition, *in vitro* experiment using LMH cells showed that NetB toxin causes cell rounding and lysing. These results challenged the view that alpha-toxin is a virulent factor in NE pathogenesis. NetB belongs to the alpha-haemolysin family of β-pore-forming toxins, and it was isolated from the *C. perfringens* type A strain in NE birds. NetB toxin-induced tissue damage may result in *C. perfringens* overgrowth (Cheung et al., 2010). In an experimental NE model, NetB negative *C. perfringens* failed to induce NE (Timbermont et al., 2011). Even though NetB has been reported as the main virulence factor for NE incidence, NetB negative bacteria were also detected in NE birds (Chalmers et al., 2008). Therefore further studies are much needed for investigating other *C. perfringens* virulence factors responsible for NE.

1.2 Predisposing factors: coccidiosis, nutrition and stress and immunosuppression

Coccidia is a protozoan parasite that propagates inside the host intestine and causes enteric disease. So far seven species of *Eimeria* (*E. maxima, E. acervulina, E. brunetti E. mitis, E. praecox, E. necatrix, and E. tenella*) have been known to infect birds (Williams et al., 2005). *Eimeria* species, such as *Eimeria maxima* and *Eimeria acervulina*, are the most known predisposing factors for NE in chickens (Al-Sheikhly et al., 1980). Even though coccidiosis and NE are associated and have the similarity of their symptoms, coccidiosis is usually prior to or during the NE phase. The *Eimeria* vaccine has been shown to increase the incidence of NE (Gholamiandehkordi et al., 2007). As a result *Eimeria* species have been commonly used as a predisposing factor for experimental reproduction of NE, either by administering a coccidia vaccine overdose or feeding live coccidia to birds in order to induce intestinal damage (Pedersen et al., 2008). Destruction of the intestinal lining by *Eimeria* may predispose the chickens to NE (Gazdzinski & Julian, 1992). Though there is no clear mechanism showing how coccidia induces NE, possible causes are proposed. Damage caused to the intestinal lumen, usually during coccidia propagation, leads to bleeding and thus the
leaked plasma proteins become a source of growth-substrate for *C. perfringens* (Van Immerseel et al., 2004). Coccidiosis induced mucus production provides a suitable environment for *C. perfringens* growth (Collier et al., 2008). *C. perfringens* is incapable of synthesizing many of the essential amino acids (Shimizu et al., 2002). Genomic sequencing data shows that *C. perfringens* cannot produce enzymes required for amino acid biosynthesis (Shimizu et al., 2004; Drew et al., 2004). Diet play a very important role in the incidence of chicken NE (Drew et al., 2004). Diets high in levels of indigestible, non-starch polysaccharides (NSP) feeds such as wheat, barley, rye and oats have shown to be a predisposing factor for NE in birds (Kaldhusdal et al., 1999). In *vitro*, extracts from wheat or barley has shown better bacterial growth compared to extracts from digested maize (Annett et al., 2002). This may be associated with the presence of arabinoxylans and β-glucans, which are not easily digested by the birds’ digestive system, however they can be used as a growth-substrate for both the microbiota and pathogenic bacteria like *C. perfringens* (Annett et al., 2002). On the other hand, NSP may create a very suitable environment for the bacteria by increasing transit time and creating digesta viscosity (Choc et al., 1999). Diets high in animal protein, such as fish or meat/bone meal, has been shown to predispose birds to *C. perfringens*-induced NE (Williams et al., 2005). In the experimental reproduction of NE, high concentration of fish meal is commonly used as a predisposing factor for NE (Cowen et al., 1987). In the lower part of the intestine, poorly or not fully digested protein can act as a substrate for the gut microflora (Timbermont et al., 2011). Fishmeal is a good source of zinc, glycine and methionine (Dahiya et al., 2006), and glycine and methionine are known to stimulate *C. perfringens* growth *in vitro* (Nakamura et al., 1968). Availability of zinc is linked with alpha-toxin production by *C. perfringens* and can prevent trypsin from destroying the alpha-toxin (Baba et al., 1992). The gizzard can be triggered by the presence of animal protein to increase the pH that favors *C. perfringens*
growth (Smith, 1965). Animal fat, such as tallow or lard, are believed to increase ileal \textit{C. perfringens} count more than plant oils (Knarreborg et al., 2002). Effects of anti-nutritional factors such as protease inhibitors, lectins, and tannins may predispose birds to NE. Trypsin inhibitors commonly found in soybean meal create a very suitable environment for bacterial growth by leaving undigested proteins in the lower gut (Clarke & Wiseman, 2007). Lectins may enhance bacterial adherence to the intestinal mucosa. Tannins can destroy intestinal tissue by strongly acting with proteins (Robins & Brooker, 2005). In addition to the chemical nature of the feed, the existence of gut microbes may depend on the physical nature of the feed (Smith & Macfarlane, 1998). Roller-mill ground wheat has been shown to minimize NE-induced death of chickens compared to hammer ground wheat (Branton et al., 1987). Coarsely ground mash may initiate HCL secretion and increase feed retention time in the gizzard and proventriculus (Engberg et al., 2004).

Stress is considered another predisposing factor for NE, this could be due to the change in the gut (McDevitt et al., 2006). Immunosuppressive diseases, such as Infectious Bursal Disease (IBD), Chick Anemia Virus and Marek’s Disease, have been suggested to exacerbate the progress of \textit{C. perfringens}-induced NE (Lee et al., 2011). Experimentally reproduced NE using IBD vaccine as a predisposing factor is evident (McReynolds et al., 2004). Chicken stressors like ammonia, physiological stress, and high stocking density have been demonstrated to suppress the immunity and to predispose chickens to NE (Tsiouris et al., 2015). They also showed the effect of environmental stress like cold stress to cause immunosuppressive induced NE. Likewise, Burkholder et al. (2008) reported that heat stress changes intestinal structure and disrupts microbiota community (Burkholder et al., 2008).
1.3. Alternative strategy for Necrotic enteritis

A probiotic is defined as “a live microbial food supplement that beneficially affects the host by improving the intestinal microbial balance” (Fuller, 1999). Probiotics have been shown to play a very important role in the host’s metabolism, immuno-stimulation, and disease protection (Edens, 2003). Probiotics can improve feed conversion efficiency, nutrient digestibility, and absorption by modulating gut microbiota (Yeo & Kim, 1997), in addition, probiotics have been shown to prevent different gastrointestinal (GI) diseases in animal production (La Ragione et al., 2004). Hence, they have been one of candidates for an alternative strategy to antibiotics. They can be given via drinking water, feed, or spray. Even though the mechanism of action is not very clear, some of the possible mechanisms can be a direct action by competing with the pathogenic bacteria for nutrient and niche establishment, or an indirect action by stimulating immune response and mucosal barrier formation. Probiotics have also been shown to change enterocyte morphology by enlarging nuclei and active impetus in cell mitosis (Kabir, 2009). Chichlowski et al. (2007) observed that probiotics to enhance villus length and cell mitosis after adding \textit{B. subtilis} to chicken feed (Chichlowski et al., 2007). The most widely used probiotics for chickens are \textit{Bacillus}, \textit{Aspergillus}, \textit{Lactobacillus Bifidobacterium}, \textit{Candida}, and \textit{Sterptomyces} (Apata, 2008). \textit{Lactobacillus} and \textit{Bacillus} are the most widely used probiotics against \textit{C. perfringens}-induced NE (Jadamus et al., 2001). Birds supplemented with probiotics showed higher antibody titer compared to control birds (Haghighi et al., 2006). They have also shown to prevent against bacterial infection by producing bacteriocins (antibacterial activity) and inhibiting bacterial toxin production (Joerger, 2003). \textit{B. subtilis} PB6 has been shown to protect birds from \textit{C. perfringens}-induced NE and to improve gut health (Jayaraman et al., 2013). \textit{B. licheniformis} has been able to inhibit \textit{C. perfringens}-induced NE in broiler chickens (Knap et al., 2010).
Vaccines are another candidate for an alternative strategy to control NE. Incidence of NE usually occurs between birds aged 2-6 weeks; this might be due to the decline of maternal antibodies at about 3 weeks of age (Ulmer-Franco et al., 2012). Comparing healthy birds with clinical NE birds, healthy birds showed higher levels of antibodies (IgY) against both NetB and alpha toxin (Lee et al., 2012). This observation suggests the importance of these antibodies on protecting against necrotic enteritis. Several studies have been conducted on preventing NE using vaccines. Some of them include inactivated toxins, live bacteria, DNA vaccines, and other proteins. These vaccines have been administrated through feed, water, spraying on hatchery, and injecting in ova (Sharma, 1999). Despite reports of partial protection by these vaccines, there is still no effective commercial vaccine against *C. perfringens*-induced NE.

1.4. Microbiota and its metabolites

Microbiota have been shown to prevent and treat different diseases. Microbiota transferred from adult healthy birds to 2-day old birds have shown to protect against cecal colonization of *Salmonella infantis* (Rantala & Nurmi, 1973). Humans GI diseases, such as recurrent *C. difficile* infection, were successfully treated by microbiota transplantation (Anderso et al., 2012; Lawley et al., 2012). Microbiota uses different mechanisms to prevent and treat diseases in animals. One of them is competitive exclusion (CE). The term CE refers to that microbiota is competing either for nutrient or physical attachment (colonization) with the pathogenic bacteria physically or chemically. For example, Zinc is an important mineral for both the microbiota and the pathogenic bacteria. Healthy beneficial microbiota prevents young chicks from pathogenic bacterial colonization by competing for nutrients in the gut (Joerger et al., 2003; Schneitz et al., 2005). Another CE mechanism is bacteriocin production. Bacteriocin secreted from the microbiota has bactericidal property, hence it can be used for both prevention and treatment against pathogenic
bacteria (Caly et al., 2015). The gut microbiota play a crucial rule in the maturation and
development of the host digestive system including intestinal epithelium and immune system, and
this is mainly mediated by lymphoid organ development, antimicrobial peptide and
immunoglobulin A production, and lymphocyte activation and differentiation (Sommer &
Bäckhed, 2013). Forder et al. (2007) demonstrated the effect of microbiota on goblet cell and
mucosal cell architecture after hatching. Comparing with germ free animals, the conventionally
grown animals have been shown better development in intestinal morphology such as mucus layer,
epithelial layer, lamina propria, villi length and crypts depth (Deplancke & Gaskins, 2001;
Abrhams et al., 1963). In addition, the beneficial bacteria have been shown strong effects on the
intestinal T cells repertoire and cytokines expression (Mwangi et al., 2010).

Gut microbiota plays an important role in the growth and the performance of the animal. Even though it is controversial whether microbiota penetrates the egg and inhabits the embryo before hatch, many studies suggest the important effect of feed, water, hatching environment and transportation on the microbiota profile and colonization in the post-hatch chickens (Kers et al., 2018). Gut microbiota and their metabolic products improve nutrient digestion, absorption, and metabolism as well as performance of the broiler chickens. This is believed to be mainly through producing short chain fatty acid (SCFAs: acetate, propionate, butyrate, and lactate), amino acids, and vitamins B and K (Ewing & Cole, 1994; Yadav & Jha, 2019). SCFAs are produced through fermentation of indigestible non-starch polysaccharides and other polysaccharides; they provide energy to the intestinal epithelial cell, increase villus height, and increase absorption (De Vadder et al., 2014). This has been supported by the research using germ-free and conventional mice. The germ-free mice have shown less villus thickness, intestinal surface area and impaired microvilli
growth compared to the conventional mice (Deplancke & Gaskins, 2001). In addition, SCFAs have also play a role in glucose regulation and energy and lipid metabolism (Den Besten et al., 2013).

1.5. Microbiota metabolites of bile acids in health and diseases

Bile acids are amphibiotic steroids synthesized from cholesterol in the liver by hepatocyte (Chiang & Li, 2009). Because bile acids are more soluble in water than cholesterol, synthesizing bile acid is the main route of cholesterol elimination from the body (Cohen, 2008). Bile consists of many components such as bile salts, water, cholesterol, phospholipids, and bilirubin; however, bile acids are the principal constituents of bile (Boyer, 2013). In addition to their importance in digestion of dietary cholesterol, lipids, fat-soluble vitamins and other essential nutrients, bile acids also play a vital role in their metabolism and regulation, transportation and control signaling events in liver regeneration (Chiang, 2013). Bile acids are generally divided into primary and secondary bile acids. Primary bile acids are cholic acid and chenodeoxycholic acid, and they are synthesized from cholesterol by hepatocytes in the liver, and they are then further conjugated with two amino acids, taurine or glycine, to become conjugated primary bile acids (Chiang, 2013). Conjugation increases bile acid's water solubility and makes them physiologically efficient for fat digestion. Before they are secreted to the duodenum via the bile duct, bile acids are stored in the gallbladder (Hundt & Young, 2019). Bile acid secretion is triggered in response to cholecystokinin secreted from the duodenum after food consumption. In response to this, the gallbladder contracts and releases bile acids to the intestinal lumen (Hofmann, 1999). In human, about 95% of the released bile acids are reabsorbed in the small intestine ileum and transported to the liver through enterohepatic circulation (Dawson & Karpen, 2015). The remainder of the bile acids are excreted via feces (A F Hofmann & Hagey, 2008; Ridlon et al., 2006). The excreted bile acids are replaced by synthesizing new bile acids in the liver, and hence the total quantity of the bile salts is maintained.
Bile acid biosynthesis occurs via two pathways, the 'classical' and 'alternative' pathways (Chiang & Li, 2009). The classical or neutral pathway occurs in the liver hepatocyte and represents the majority of the primary bile acid synthesis. The classical pathway is initiated with the 7-α hydroxylation of cholesterol by cholesterol 7-α hydroxylase (CYP7A1), and the next 12-α hydroxylation is followed by multiple steps resulting in the synthesizing of the primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA) through the subsequent enzymatic action of CYP8B1 and CYP27A1 (Thomas et al., 2008). Failure or reduction in bile acid synthesis through the classical pathway is compensated by the alternative pathway (Pullinger et al., 2002). The alternative pathway is initiated with the hydroxylation of the cholesterol side-chain by sterol 27 hydroxylase (CYP27A1) and produces CDCA via CYP7B outside the liver. The ratio of CDCA to CA is determined by the expression of CYP8B (Chiang & Li, 2009). Gut microbiota deconjugates and 7α-dehydroxylates the primary bile acids and converts them to secondary bile acids. They are called secondary bile acids since they are formed due to the action of gut microbiota. Secondary bile acids include deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). CA and CDCA are converted to DCA and LCA, respectively (Devlin & Fischbach, 2015; A F Hofmann & Hagey, 2008). Both conjugated and unconjugated bile acids are absorbed from the intestine and return to the liver via hepatic portal circulation (Dawson & Karpen, 2015). After returning to the liver, these bile acids inhibit new bile acid synthesis by suppressing the rate-limiting enzyme CYP7A1. Liver then conjugates the unconjugated bile acids and secretes them again into the intestine via the bile duct (Gurantz et al., 1991).

In addition to their importance in lipid digestion and absorption, bile acids also play a critical role as signaling molecules (ligands) in gene expression, lipid, and glucose metabolism and bile acid synthesis through activating the bile acid receptors (Chiang, 2013). Bile acids are directly involved
in their own synthesis through regulating gene expression (T. Li & Chiang, 2013). Bile acid receptors farnesoid X receptor (FXR) and G-protein-coupled receptor-1 (GPBAR-1 or TGR5) are found in the liver and intestine (Maruyama et al., 2002; Duboc et al., 2014). Farnesoid X receptors (FXR) are nuclear receptors expressed at a high level in the liver and intestine (Makishima et al., 1999; Schaap et al., 2014). CDCA is considered as a natural ligand for FXR. Bile acid production is inhibited or activated based on the intestinal levels of bile acids. One of the main functions of FXR activation is to suppress CYP7A1 in the hepatocyte (Gupta et al., 2001). When the intestinal level of bile acids is high, a negative feedback pathway inhibits the production of new bile acids (Heuman et al., 1989; Chiang, 2002).

In addition to their importance in host nutrition, metabolism, and immune homeostasis, gut microbiota plays a vital role in the metabolism of bile acids (González-Regueiro et al., 2017). Studies have shown that the population and composition of gut microbiota are affected by the content and nature of bile acids (Kakiyama et al., 2013). Some of the most common human gut microbiota involved in bile acids metabolism are Bacteroides, Bifidobacterium, Clostridium, Lactobacillus, Eubacterium, and Escherichia (Elkins et al., 2001; B. V Jones et al., 2008). Rats fed CA diet have shown to increase the phylum Firmicutes significantly and to reduce the population of Bacteroidetes and Actinobacteria compared to the control group (Islam et al., 2011). At the distal part of the intestine, gut microbiota converts primary bile acids, CA and CDCA, into secondary bile acids of DCA, LCA and UDCA (Devlin & Fischbach, 2015; A F Hofmann & Hagey, 2008). Primary bile acids undergo three main steps to be converted into secondary bile acids: deconjugation, 7α-dehydroxylation, and 7α-dehydrogenation. Deconjugation of primary bile acids is mediated by the enzyme bile salt hydrolase (BSH), and this leads to the cleavage of the amino acid side chain taurine or glycine group (Ridlon et al., 2006). Deconjugation increases
hydrophobicity and toxicity of bile acids to microbiota. Dehydroxylation (removal of OH group to form 7-deoxy bile acids) is done by bacterial dehydratases of the anaerobic microbiota; hence they convert CA to DCA (3α, 12α-dihydroxy-5β-cholanoic acid) and CDCA to LCA (3α-hydroxy-5β-cholanoic acid (Monte et al., 2009; Gupta et al., 2001). In humans, this is mainly done by the genus Clostridium (Buffie et al., 2015). UDCA is commonly found in less amounts in humans and is synthesized by the action of hydroxyl steroid dehydrogenases (HSDHs) enzymes of the gut microbiota after oxidation and epimerization of the 3-, 7- or 12-hydroxyl groups of bile acids. Epimerization is a reversible process. Clostridium, Bacteroides, Eubacterium, Peptostreptococcus, and Escherichia coli are some of the enteric species that can undergo oxidation and epimerization of hydroxy groups. These secondary bile acids have been shown to modulate the gastrointestinal bacterial population; this is associated with the detergent property of bile acids. Primary and secondary bile acids are considered as signaling molecules of (FXR) and G-protein-coupled (GPBAR1) receptors, and together they are called bile acid-activated receptors (BARs) (Maruyama et al., 2002; Duboc et al., 2014). BARs signaling has been shown to be modulated by gut microbiota. In return, the bile acids also determine the composition and population of gut microbiota. Even though the mechanism of how BSH protects the microbiota is not fully studied (unconjugated bile acids are more hydrophobic and toxic), BSH enzymes produced by the microbiota are considered as a mechanism for protection against the toxic effect of bile salts (Begley et al., 2005). BSH hydrolyzed amino acids are suggested to have a nutritional advantage for the bacteria (Begley et al., 2005). In vitro studies have shown that bile salts change cell walls and cell membranes of Lactobacilli and Bifidobacteria. Deconjugation of primary bile acids by BSH is the first step for 7a β dehydroxylation. Bacteria species belonging to Clostridia are responsible for seven a β dehydroxylation (Wells et al., 2003). Intestinal microbiota plays a
vital role in primary and secondary bile acids production and biotransformation through the FXR receptor. In addition to the biotransformation to secondary bile acids, gut microbiota has also been shown to inhibit bile acid production through alleviation FXR inhibition in the ileum (Sayin et al., 2013).

In addition to their significant role in lipid digestion, metabolism, and signaling, bile acids have been shown to treat both infectious and metabolic diseases (T. Li & Chiang, 2014). Mice with obstructed bile ducts have shown proliferation and invasion of bacteria into the intestine, and this action was recovered through oral administration of bile acids (Makishima et al., 2002). Dysbiosis has been associated with infectious and metabolic diseases (Carding et al., 2015). Dysbiosis caused by an alteration in secondary bile acids affects host glucose, lipid, and drug metabolism, suggesting the potential use of bile acids as a therapeutic target for treating metabolic disease (Chiang & Li, 2009). Disruption of the bile acid profile induces inflammation and leads to the development of the gastrointestinal disorders, such as *C. difficile* infection, irritable bowel syndrome, inflammatory bowel diseases, and metabolic syndrome (Sartor, 2011; Schubert et al., 2014; Mayer, Savidge, & Shulman, 2014). Hence modulation of gut microbiota and bile acid profiles can be an effective therapeutic approach for the treatment of gastrointestinal diseases. Secondary bile acids have been shown to prevent and treat against pathogenic microorganisms and other metabolic disorders. For instance, UDCA is used in the treatment of cholesterol gallstones (Guarino et al., 2013). The Food and Drug Administration (FDA) approved UDCA for the treatment of primary biliary cirrhosis (Paumgartner, 2003). On the other hand, increasing the amount of secondary bile acids is associated with colon cancer and cholesterol gall stones in human (Farhana et al., 2016).

*C. difficile* infection is one of the nosocomial infections in the US (Ananthakrishnan, 2011). *C. difficile* is a Gram-positive, spore-forming and anaerobe bacterium. *C. difficile* vegetative induces...
inflammation and diarrhea by producing enterotoxins. Growth and germination of *C. difficile* have been shown to be affected by the presence of bile acids. Primary bile acids taurine–conjugated CA, taurocholic acid, has shown to induce germination of *C. difficile*; hence it is a normal ingredient of *C. difficile* growth media (Wilson, 1983). Primary bile acid CDCA has been shown to inhibit *C. difficile* spore germination (Sorg & Sonenshein, 2009; Sorg & Sonenshein, 2010 ;). On the other hand, *in vitro* experiment has shown that secondary bile acid LCA and UDCA inhibit *C. difficile* germination (Palmieri et al., 2018; Theriot et al., 2014). *In vivo*, antibiotic-treated mice have shown a lower proportion of secondary bile acids and higher growth of *C. difficile* than untreated mice, demonstrating the importance of secondary bile acids in inhibition and growth of *C. difficile* (Theriot et al., 2014). The normal biotransformation of primary bile acid to secondary bile acid by the gut microbiota was disrupted in patients with recurrent *C. difficile* infection. Recently, fecal microbiota transplantation has been shown to successfully treat *C. difficile* infection by restoring the normal composition of the gut microbiota. This transplanted microbiota has shown to restore fecal bile acids by regulating gut microbiota (Weingarden et al., 2014).
Figure 1. Figure 1.1 Flow chart demonstrates the structures of primary and secondary bile acids with their synthetic pathways. The bile acid chemical structures were obtained from https://en.wikipedia.org/wiki/Bile_acid and chart was then drawn based on this report (Ridlon et al., 2006).
1.7 Reference


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Chapter II:

Evaluation of Microbial Metabolic Product Deoxycholic Acid on Chicken Necrotic Enteritis
2.1 Abstract

The reemerged necrotic enteritis (NE), caused by coccidiosis and *C. perfringens* infection, costs $6 billion every year worldwide. Few effective antimicrobial alternatives are available to control the disease. The objective of this study was to evaluate prevention and treatment of NE using microbiota metabolic product secondary bile acid deoxycholic acid (DCA) in drinking water. One-day-old birds were tagged and placed in floor pens. In experiments 1 and 2, the birds were infected with *Eimeria maxima* at d 18 and *C. perfringens* at d 23 and 24 and the birds were euthanized at d 26. In experiment 3, the birds were infected with *Eimeria* at d 20 and *C. perfringens* at d 25 and 26 and euthanized at d 27. DCA was administrated in drinking water at 1.91, 0.95, or 0.48 mM. Samples of small intestinal content and tissue were collected for isolating DNA, RNA or histopathology. In experiments 1 and 3, birds experienced mild and severe body weight gain (BWG) reduction respectively. Because of minimal body weight alteration in *Em* and NE birds compared to non-infected birds in experiment 2, no tissue were collected for histopathology and molecular biology assays. NE birds in experiment 1 developed mild NE of subclinical signs and histopathology, while NE birds in experiment 3 suffered acute NE. Remarkably, 1.91 mM DCA attenuated the BWG reduction in experiments 1 and 3. Consistently, DCA at drinking water reduced NE-induced intestinal histopathology in birds of experiments 1 and 3. Mechanism studies revealed that DCA reduced NE-induced expression of inflammatory gene *Infγ* in experiment 1. Similarly, DCA reduced NE-associated *C. perfringens* luminal colonization elevation in experiment 1. Using targeted metabolomics (LC-MS/MS) of bile acids, we found that NE is associated with reduction of total bile acids in ileal content, an effect attenuated with DCA supplementation in drinking water in experiment 1. In conclusion, microbial metabolic product DCA in drinking water prevents NE-induced BWG reduction and histopathology, perhaps through
reducing *C. perfringens* colonization and/or bile acid reduction. These findings may offer an alternative strategy to use DCA for NE prevention and treatment.
2.2 Introduction

Necrotic enteritis (NE) has reemerged as one of the prevalent intestinal diseases in the poultry industry because of restricting usage of prophylactic antibiotics under consumer preferences and regulatory pressures. It is estimated that NE costs $6 billion every year worldwide (Wade and Keyburn, 2015). The main causative pathogens of NE are a spore-forming, anaerobic, and Gram-positive bacterium *Clostridium perfringens* and coccidia *Eimeria maxima* or *E. acervulina*. Acute NE by *C. perfringens* infection causes sudden death in birds of up to 50% mortality (Caly et al., 2015). Subclinical NE is more common, and no obvious clinical signs are observed often without peak mortality.

The withdrawing of antimicrobials in poultry production, however, is facing a number of challenges, because antimicrobial free feeding regiments have affected the poultry industry by elevated productivity loss and diseases, such as NE. Dissection of dead or severely ill NE birds show that intestines are ballooned with gas, fragile and contain a foul-smelling brown fluid (Timbermont et al., 2011). At the cellular level, intestinal tract of acute NE birds displays severe small intestinal inflammation as infiltration of massive immune cells into lamina propria, villus epithelial line necrosis, and crypt hyperplasia (Al-Sheikhly and Truscott, 1977; Long et al., 1974; Wang et al., 2019). Progresses have been made toward understanding risk factors influencing the outcome of NE such as *C. perfringens* virulence, coccidiosis, and feed (Prescott et al., 2016). However, it remains largely unresolved on NE pathogenesis, and little progress has been made on the strategies of antibiotic alternatives to prevent and treat NE.

*C. perfringens* colonizes in the intestinal tract among normal chicken microbiota. The presence of a high dose of *C. perfringens* in the intestine does not sufficiently induce NE and its count alone does not associate with NE (Craven, 2000; Kaldhusdal et al., 1999; Pedersen et al., 2003).
Pathogenic *C. perfringens* is thought to drive the development of NE with various toxins. Different strains of *C. perfringens* produce a variety of toxins such as alpha (CPA, type A), enterotoxin (CPE), necrotic enteritis B-like toxin (NetB), and others (Navarro et al., 2018). Among them, *C. perfringens* isolated from NE birds are mainly type A strains. Researchers in Australia have reported that NetB but not CPA induced NE in chickens (Keyburn et al., 2008). However, NE does not have strong association with NetB positive *C. perfringens* in the United States (Martin and Smyth, 2009).

Microbiota composition is altered in NE birds compared to healthy birds (Antonissen et al., 2016). The human and animal intestine harbors up to trillions of microbes, and this intestinal microbiota regulates various host functions such as the intestinal barrier, nutrition and immune homeostasis (Belkaid and Hand, 2014; Caricilli et al., 2014; Subramanian et al., 2014; Sun and Jia, 2018). At the gut level, microbiota transplantation has shown tremendous success against recurrent *Clostridium difficile* infection (Silverman et al., 2010). The production of secondary bile acids by *C. scindens* is found to inhibit *C. difficile* colonization and infection (Buffie et al., 2015). Bile acids synthesized and conjugated in the liver, such as tauro- or glycol-cholic acids (TCA or GCA) and tauro- or glycol-chenodeoxycholic acids (TCDDCA or GCDDCA), are metabolized in the intestine by the gut microbiota through deconjugating (e.g. CA or CDCA). They are then transformed into secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA), or ursodeoxycholic acid (UDCA) (Ridlon et al., 2006). Majority of bile acids (>95%) are effectively absorbed in the intestine (Ridlon et al., 2006). Bile acids have been reported in association with a variety of chronic diseases (Bernstein et al., 2011; Ma and Patti, 2014), but new evidences shed light on the beneficial property of secondary bile acids in health and diseases, such as gut motility (Yano et al., 2015), and *C. difficile* infection (Buffie et al., 2015).
Anaerobic microbiota and its metabolite DCA prevent *Campylobacter jejuni*-induced intestinal inflammation (Sun et al., 2018) in mice and the bacterial colonization in chickens (Alrubaye et al., 2019). More recently, we found that DCA supplemented in feed prevented NE-induced acute intestinal inflammation and body weight gain reduction (Wang et al., 2019). In the study, decreased feed intake in NE birds posed an obstacle to effectively delivering sufficient dietary DCA to the birds. To address this issue, in these current experiments, different doses of DCA in drinking water were used to prevent and treat subclinical and acute NE. The results from this study will help the understanding of NE pathogenesis and assist in developing new antimicrobial alternatives against NE.
2.3 Material and method

Chicken experiments

All the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas. The experiments were conducted at JKS Poultry Health Laboratory at University of Arkansas at Fayetteville. Day old broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR). They were received, neck-tagged and randomly assigned to floor pens in an environmentally-controlled house. No medication was added to the feed. Feed and water were supplemented *ad libitum* and temperature was maintained according to their age, 34°C for the first 5 days and then gradually decreased to 23°C until the final day 26 or 27. The broiler chicks were fed a corn-soybean meal-based starter diet for the first 0-10 days of age and a grower diet during 11-27 days of age. Because gross necrotic lesion was often observed in upper ileum in this NE model, the ileal tissue and digesta samples from all sacrificed birds were collected for RNA and DNA analysis. Below were the three separated chicken experiments.

Experiment 1

Day old birds were placed in 4 floor pens with 16 birds per pen. DCA at 1.91 mM was administrated in drinking water from d 16 to 26 as a prevention approach. The birds were then gavaged with 20,000 sporulated oocytes/bird *E. maxima* at d 18 as described before (Wang et al., 2019). An aliquot of frozen *C. perfringens* was cultured in TSB and sodium thioglycolate overnight for the NE challenge study, and it was serially diluted and plated on tryptic soy agar plus sodium thioglycolate for enumerating colony forming unit (CFU). The broiler birds were gavaged with $10^9$ CFU/bird *C. perfringens* at d 23 and 24 as described before (Wang et al., 2019). Chicken body weight was individually measured at d 0, 18, 23, and 26. Bird health status was monitored
daily after the pathogen infection. Six birds with average BW of each group were sacrificed at d 23 and 26. Intestinal tissues were collected for histopathological analysis from proximal ileal tissue of about 10 cm, starting close to Meckel’s diverticulum. The samples then were flushed with PBS buffer (pH 7.6) and then excised longitudinally and then positioned the mucosa side up on the small pieces of blotting paper. The intestinal tissue was then Swiss rolled and fixed in 10% buffered formalin solution, later dehydrated and embedded in paraffin for histological processing.

Experiment 2

Day-old birds were placed in 6 floor pens with 16 birds per pen. DCA at 1.91 mM was administrated in drinking from d 16 (NE prevention 1 (prev1)), 20 (NE prevention 2 (prev2)), and 23 (NE treatment (trt)). The birds were infected with *E. maxima* and *C. perfringens* as described above. The birds were individually weighted at d 0, 18, 23, and 26 as described above. Bird health status was monitored daily after the pathogen infection. Because minimal body weight alteration in *Em* and NE birds compared to noninfected birds, no tissue were collected for histopathology and molecular biology assays.

Experiment 3

Day-old birds were placed in 7 groups (2 floor pens/group and 16 birds/pen). DCA was administered in drinking water at 1.91, 0.95 or 0.48 mM from d 17 (prev), or 1.91 mM at d 25 (NE trt). Coccidiosis vaccine was purchased from Huvepharma, (ADVENT) and the vaccine contains live oocysts of *E. acervulina, E. maxima and E. tenella*. Broiler chicks were challenged with *E. maxima, E. acervulena and E. tenella* at 60,000, 58,000 and 17,500 sporulated oocysts/bird, respectively, at d 20 to induce coccidiosis. Then the birds were gavaged with $10^9$ CFU/bird *C. perfringens* at d 25 and 26 as described above. Individual chicken body weight was measured at d
0, 20, 25, and 27. The bird health status was monitored daily after the pathogen infection. Five birds with representative BW of each group were sacrificed at d 25 and 27. Intestinal tissues were collected for histopathological analysis from the proximal ileal tissue as described above.

**Histopathology evaluation**

Intestinal Swiss-Rolled samples were processed at the Histology Laboratory in the Department of Poultry Science at the University of Arkansas at Fayetteville. Briefly, the tissue was dehydrated and embedded in paraffin. The section was cut at 5 μm thick using a rotary microtome. The slide was then stained with standard Hematoxylin and Eosin (H&E) to determine for histological morphology. Photographs were acquired using a Nikon TS2 fluorescent Microscope system. Ileal inflammation was scored as described before (Wang et al., 2019). Briefly, each Swiss-Rolled slide was divided into 4 areas and scored. Total histopathological scores were then calculated by adding the 4 area scores. The following score scales were used as described before (Wang et al., 2019). Briefly, score 0: no inflammation and villi and crypt intact; score 1: small number infiltration cells in laminar propria of villi and crypts or villi minimally shortened; score 2: more extensive infiltration cells in laminar propria of villi and crypts, villi shortened >¼ and edema, or crypt hyperplasia; score 3: pronounced infiltration cells in laminar propria of villi, crypts, submucosa, and muscularis, villi shortened >½ and edema, or crypt hyperplasia and regeneration; and score 4: necrosis, villus diffuse, ulcers, crypt abscesses, or transmural inflammation (may extend to serosa).

**Real time RT-PCR of mRNA gene expression and C. perfringens quantification**

Total RNA from ileal tissue was extracted using TRIzol as described before (Wang et al., 2019). cDNA was prepared using M-MLV (NE Biolab). mRNA levels of proinflammatory genes were determined using SYBR Green PCR master mix (Bio-Rad) on a Bio-Rad 384-well Real-Time PCR
system and normalized to Gapdh. To quantify C. perfringens intestinal luminal colonization or tissue invasion, ileal digesta or tissue were weighed, bead-beated, and extracted for DNA using a phenol-chloroform method as described before (Wang et al., 2019). C. perfringens in the digesta and tissue was quantified using specific C. perfringens 16S rDNA primers by the qPCR. The PCR reactions were performed according to the manufacturer’s recommendation. The following gene primers were used:

*Cp16S* _forward:* 5’- CAACTTGGGTGCTGCATTCC-3’; *Cp16S* _reverse:* 5’- GCCTCAGCGTCAGTTACAG-3’; *Mmp9* _forward:* 5’-CCAAGATGTGCTCACCAAGA-3’; *Mmp9* _reverse:* 5’-CCAATGCCTTCTCAAT-3’; *Litaf* _forward:* 5’- AGATGGAAGGGAATAGAACC; *Litaf* _reverse:* 5’-GACGTGTCACGATCATCTGG-3’; *Il1β* _forward:* 5’-GCATCAAGGGCTACAAGCTC-3’; *Il1β* _reverse:* 5’- AGCCGGCTACAAACACATA -3’; *Infγ* _forward:* 5’- CCAGGTTGCTGCTCTACTCC-3’; *Infγ* _reverse:* 5’-AGCCGCACATCAAACACATA -3’; *Gapdh* _forward:* 5’-GACGTGCAGCAGGAACACTA-3’; *Gapdh* _reverse:* 5’-CTTGGACTTTGCCAGAGAGG-3’.

Gene expression of fold change was calculated using ΔΔCt method and Gapdh as internal control.

**Quantification of ileal bile acids using targeted metabolomics**

In experiment 1, the ileal digesta was collected and the bile acid composition was analyzed using multiple reaction monitoring (MRM) mass spectrometry in the Statewide Mass Spectrometry Facility at the University of Arkansas at Fayetteville. Intestinal bile acids were extracted using chloroform/methanol. To identify and quantify bile acids, MRM methods have been developed for tauro- and glyco-cholic acid (TCA/GCA), tauro- and glyco-chenodeoxycholic acid (TCDCA/GCDCA), cholic acid (CA), chenodeoxycholic (CDCA), deoxycholic acid (DCA),
lithocholic acid (LCA), ursodeoxycholic acid (UDCA) isotopically labeled (some were with deuteriums and some were with five deuteriums) and unlabeled standards. The methods were fully optimized using data acquisition software attached to Shimadzu UPLC-20A/LC-30A coupled with Shimadzu 8050 triple quadrupole mass spectrometer at their respective retention times. Once the methods were optimized, the samples with isotopically labeled internal standards were analyzed along with standards with varying concentrations. Calibration curves with internal standards were used to estimate bile acid concentration. Use of internal standard accounts for sample loss during the extraction procedure. LC-MS/MS analysis was performed in negative-ion mode. Methodology is quite similar to what has been described before (Diaz Perez et al., 2019). LC separation was performed using a C18 column. The flow rate was 0.3 mL/min and sample volumes of 1µL were injected. The three most intense multiple reaction monitoring (MRM) fragments for each bile acid standard were first fully optimized at their respective retention time windows using Shimadzu Lab solution software as described before. From the three MRM channels, the MRM event corresponding to the most intense fragment ion was used as the “quant” ion and the other two fragment ions were used as reference ions for identification purposes. Reference ion ratios were calculated compared to the “quant” ion, and unknown samples were required to meet within 30% of the reference ion ratio of the standard.

**Statistical Analysis**

Data were first analyzed by One-way ANOVA for significant difference and then *t*-test using Prism 7.0 software. Experiments were considered statistically significant if *P* values were < 0.05.
2.4 Results

DCA reduced NE-induced body weight gain reduction

In experiment 1, the birds infected with *E. maxima* and *C. perfringens* showed subclinical NE signs with mild diarrhea and no mortality. There was no difference in daily body weight gain (BWG) between groups of birds during the uninfected phase from d 0 to 18 and during the *E. maxima* infection phase (*Em*) from d 18 to 23 (Figure 2.1). During necrotic enteritis phase (NE) from d 23 to 26, NE birds showed 26% BWG reduction (76 vs. 56 g/day/bird, P = 0.03) compared to uninfected birds. Notably, DCA at 1.91 mM in drinking water trended to restore the BWG reduction compared to NE birds (71 vs. 56 g/day/bird, P = 0.07).

To validate these results of drinkable DCA against NE, we ran experiment 2 with 6 groups of uninfected, *Em*, NE, NE+ DCA 1.91 mM from d 16 (NE prevention 1 (prev1)), NE+ DCA 1.91 mM from d 20 (NE prevention 2 (prev2)), and NE+ DCA 1.91 mM from d 23 (NE treatment (trt)). The NE birds did not show any clinical signs of NE. Consistent with the weak clinical signs, body weight gain between the groups were not significant (Figure 2.2). The minimal NE disease in this experiment might be due to the inability of *E. maxima* to induce coccidiosis.

Next, to repeat the results of drinkable DCA against NE, we ran experiment 3 with 7 duplicated groups of uninfected, *Em*, NE, NE+ DCA 0.48 mM from d 16 (DCA prevention low), NE+ DCA 0.95 mM from d 16 (DCA prevention medium), NE+ DCA 1.91 mM from d 17 (DCA prevention high) and NE+ DCA 1.91 mM from d 23 (NE trt). Consistently, BWG between groups of birds was not different before infection from d 0 to 20 (Figure 2.3). In contrast with experiments 1 and 2, the birds in this experiment were challenged with higher doses of a mixture of *E. aceralina*, *E. maxima*, and *E. tenella*. As a result, the high dose of coccidia infection induced strong coccidiosis with severe watery diarrhea, morbidity of birds, and body weight gain reduction (Figure 2.4).
Specifically, at *Em* phase of d 20 to 25, coccidia-infected birds lost more than 41% of BWG compared to non-infected birds (36 vs. 61 g/day/bird, *P* = 0.0001), while DCA prevention and treatment failed to improve BWG. At the NE phase from d 25 to 27, NE birds severely lost BWG compared to non-infected birds (-7 vs. 67 g/day/bird, *P* =0.0001). Notably, DCA high dose (prevention) of 1.91 mM in drinking water was able to recover the BWG (26 vs. -7 g/day/bird, *P* = 0.0136). Together, these results suggest that the high dose of 1.91 mM DCA as a prevention approach is able to increase BWG in subclinical and acute NE.

**DCA alleviated the NE-induced intestinal inflammation**

To evaluate how NE and DCA impact BWG, we then sought to examine the underlying histopathological alteration. The ileal tissues were collected and H&E histology slides were prepared. In experiment 1 of subclinical NE, upon the examination of the histopathology, the small intestine of NE birds showed intestinal inflammation such as shortening of villi, hyperplasia of crypt, and infiltration of inflammatory cells into the intestinal lamina propria comparing with uninfected birds. Remarkably, DCA at 1.91 mM in drinking water reduced the histopathological lesions and scores (7.2 vs 12, *P* =0.0173) compared to NE birds (Figure 2.5A and B).

To further evaluate the effect of DCA against acute NE, histology slides in experiment 3 were prepared. Consistent with the previous report (Wang et al., 2019), the *Em* birds showed intestinal inflammation and NE birds extended the inflammation, an effect attenuated by 1.91 mM DCA in drinking water as a prevention (Figure 2.6A). As a result, NE birds with a high dose of DCA in drinking water showed reduced histopathological scores (Figure 2.6 B) compared to NE birds (7.2 vs 16, *P* =0.0073). Interestingly, DCA prevention at 0.48 and 0.95 mM or DCA treatment at 1.91 mM reduced histopathological lesions and scores to a lesser extent. Together, the results suggest
that DCA at a high dose (prevention) in drinking water was able to prevent subclinical and acute NE-induced intestinal inflammation.

**DCA alleviated inflammatory mediators**

Inflammation is an important pathological feature of necrosis (Zitvogel L, 2010); we next evaluated the role of DCA on host inflammatory responses. We evaluated the expression of proinflammatory mediator genes in chicken ileal tissue in experiment 1 of subclinical NE using Real-Time PCR. mRNA in ileal tissue was isolated and cDNA was prepared. mRNA levels of proinflammatory genes of *Infγ*, *Litaf* (*Tnfα*), and *Il1β* were determined using SYBR Green PCR master mix system and normalized to *Gapdh*. Interestingly, NE induced proinflammatory gene *Infγ* mRNA accumulation by 4 folds compared to that in uninfected birds (Figure 2.7). Importantly, DCA reduced the inflammatory mediator gene expression by 75 % compared to that in NE birds.

**DCA reduced *C. perfringens* luminal colonization in small intestine**

The reduction of inflammatory response by DCA in drinking water could be attributed to less *C. perfringens* colonization in ileum. To examine this possibility, we measured *C. perfringens* intestinal luminal colonization in ileal lumen. The ileal digesta were weighed, bead-beated, and extracted for DNA using a phenol-chloroform method as described before (Wang et al., 2019). *C. perfringens* in the digesta was quantified using real time PCR targeting specific *C. perfringens* 16S rDNA primers (Wang et al., 2019). Interestingly, NE increased *C. perfringens* luminal colonization by 2 logs compared to noninfected birds (Figure 2.8). Remarkably, DCA significantly reduced *C. perfringens* luminal colonization in the small intestine compared to NE birds.
DCA restored NE-induced bile acid reduction in ileum

Bile acids are important host-derived microbial metabolites and play an essential role in health and diseases (Sun and Jia, 2018). Limited knowledge is available on how intestinal bile acids are changed during NE. We reasoned that DCA in drinking water might influence the status of the ileal bile acid pool. To address this possibility, we performed targeted metabolomics on 9 bile acids using ileal content samples in experiment 1. Notably, NE strongly reduced total bile acid levels (7000 vs. 2000 nmol/g digesta, P= 0.0127) (Figure 2.9A). Remarkably, DCA in drinking water increased the total bile acid level to 5000 nmol/g. Furthermore, DCA in drinking water modified bile acid composition. As showed in Figure 2.9B, DCA in drinking water increased DCA proportion from 0.1 to 51% in the expense of TCDA (from 42 to 34%), CDCA (from 34 to 5%), TCA (from 15 to 6%), and CA (from 6 to 1.5%). These results suggest that NE reduces ileal bile acid pool and DCA is able to restore the bile acid pool reduction.
2.5 Discussion

We previously reported that DCA supplemented in feed reduces acute NE (Wang et al., 2019). In that experiment, the reduced feed intake in both NE and DCA-fed chickens raised the concern of the effectiveness of dietary delivery of DCA. To address this issue, we conducted three follow-up experiments to use alternative delivery approach of DCA in drinking water. We also investigated the possibility to treat NE using drinkable DCA in water. Interestingly, the birds in the current study experienced three levels of NE from none, subclinical, to acute ileitis, in association with increased severity of coccidiosis respectively. Importantly, DCA at 1.91mM drinking water prevented both subclinical and acute NE, showing attenuated body weight gain reduction and intestinal inflammation. DCA with lower doses, or as treatment, failed to reduce NE and body weight gain reduction.

One of the interesting observations in this study was that NE outcome was exclusively dependent on coccidiosis severity. It is well known that NE outbreak is dependent on various predisposed factors such as coccidiosis, feed, and *C. perfringens* virulence. Various *C. perfringens* strains produce a number of toxins such as alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), necrotic enteritis B-like toxin (NetB), and others (Navarro et al., 2018). Among them, it is reported that NetB but not CPA induces NE in chickens (Keyburn et al., 2008). However, NE has not been strongly associated with NetB positive *C. perfringens* in the United States (Martin and Smyth, 2009). In our experiment 1, because of the old *E. maxima*, the birds experienced weak coccidiosis, showing mild clinical signs and body weight gain reduction. As a result, the following NE was subclinical on clinical signs, body weight gain reduction, intestinal histopathology, and inflammation. Consistently, in the second experiment, no coccidiosis and no subsequent NE were present at all because of the even older *E. maxima*. In contrast, in the third
experiment, we used fresh and higher doses of a mixture of *E. acerulina*, *E. maxima*, and *E. tenella*. As expected, severe coccidiosis was present, and the birds showed profuse watery diarrhea and severe body weight gain reduction. Consequently, the followed NE was severe. In all three experiments, we used the same dose of the same NetB positive *C. perfringens*, whereas the outcome of NE was strikingly different only because of the coccidiosis severity variations. Based on these results, it is apparent that coccidiosis severity is indispensable for the successful development of subsequent NE.

Although DCA at 1.5 g/kg diet was able to effectively prevent acute NE (Wang et al., 2019), improvement is needed to reduce cost and to increase treatment efficiency. The higher DCA dose of 1.91mM in drinking water is approximately similar to the 1.5 g/kg diet when feed intake and water consumption has a ratio of 1 to 2 (Pesti et al., 1985). The advantage of water delivery is the ease and quickness to administrate DCA at farms. In addition, we observed that water consumption of NE birds was reduced less compared to feed intake. Consistent with the previous report (Wang et al., 2019), high doses of DCA at 1.91m M drinking water was able to prevent both subclinical and acute NE. In addition, high doses of DCA at 1.91mM drinking water reduced *C. perfringens* intestinal colonization in subclinical NE birds. DCA reduces *C. perfringens in vitro* growth (Wang et al., 2019) and the reduction of NE and *C. perfringens* in NE birds was expected. The high dose of DCA in drinking water as prevention approach was administrated just before coccidia infection (d 16 or 18), while dietary DCA in the previous study was started from d 0. Both of these approaches are effective against NE and it is therefore possible to supplement DCA into the diet at later age.

It is puzzling that the medium (0.95 mM) and lower doses (0.47mM) of DCA as prevention failed to reduce acute NE. It was observed (data collection not successful) that those birds consumed
more water compared to birds with high dose DCA during NE, suggesting comparable total DCA intake between groups. This indicates that a minimal threshold concentration of DCA in drinking water is needed to effectively prevent NE. Another unexpected result is that the high dose of DCA as a treatment approach did not reduce acute NE. This result suggests that DCA takes a longer time to reduce NE possibly either to attenuate coccidiosis or to accumulate sufficient DCA in the enterohepatic circulation/cycle. Since bile acids as a treatment and prevention against disease is new, further research is much needed.

Furthermore, subclinical NE birds apparently had 67% less total bile acids in ileal content compared to that of uninfected birds, whereas the NE birds had 26% less body weight gain reduction compared to the uninfected birds. Patients with inflammatory bowel disease, especially those infected at the distal part of the ileum, often show bile acid malabsorption (Gothe et al., 2014; Vitek, 2015). Interestingly, the expression of the apical sodium/BA cotransporting polypeptide is responsible for ileal BA reabsorption and is reduced in ileal biopsies from patients with Crohn’s Disease free of any signs of inflammation (Jung et al., 2004). Although the birds in experiment 1 had a subclinical NE with no severe obvious diarrhea present, the loss of bile acids in excrete from the enterohepatic cycle in the birds might be one of the factors. Importantly, in experiment 1, DCA in drinking water was able to compensate for the loss of the total bile acid reduction, in association with reduced NE on body weight loss, intestinal histopathology, and inflammation. It remains unclear whether the protection from drinkable DCA comes from actions to specifically antagonize C. perfringens or just to restore total intestinal bile acid pool. Following up research is needed to clarify the underlying mechanism.

In conclusion, these data indicate that DCA in drinking water was able to prevent subclinical and acute NE, possibly through restoring the intestinal bile acid pool and reducing C. perfringens ileal
colonization. The finding from this study pinpoints the need to understand the bile acid metabolism in intestinal health and diseases. These discoveries could be applied to control NE and other intestinal diseases targeting the microbiome and host metabolism.
2.6 Figures

Figure 2.1  DCA reduced subclinical NE-induced body weight gain (BWG) reduction in experiment 1.

Cohort of one-day-old broiler chicks were individually tagged and placed in three floor pens (16 birds/pen). DCA at 1.91 mM was administrated in drinking water from d 16 to 26 as prevention. The chickens were gavaged with 20,000 sporulated oocytes/bird *E. maxima* at d 18 and 10⁹ CFU/bird *C. perfringens* at d 23 and 24. Bird BWG was measured at d 18 (16 birds/group), 23 (11 birds/group), and 26 (8–9 birds/group). Six birds with representative BW in each group were euthanized at d 26. All graphs depict mean ± SEM. *P < 0.05; **P < 0.01.
Cohort of day-old broiler chicks were individually tagged and placed in six floor pens (16 birds/pen). DCA at 1.91 mM was administrated in drinking water from d 16 to 26 (NE prevention (prev1)), d 20 to 26 (NE prev2), or d 23-26 (NE treatment (trt)). The birds were infected with *E. maxima* and *C. perfringens* as described in Figure 2.1. Bird BWG was measured at 18 (16 birds/group) and 26 (8–9 birds/group) days of age. All graphs depict mean ± SEM.
Cohort of day-old broiler chicks were individually tagged and placed in fourteen floor pens (2 pens/group and 16 birds/pen). DCA was administrated in drinking water from d 17 to 27 as prevention at 0.48, 0.95, or 1.91 mM, or as treatment from d 25 to 27 at 1.91 mM. Broiler chicks were challenged with *E. maxima*, *E. acervulena* and *E. tenella* (60,000, 58,000 and 17,500 sporulated oocysts/bird, respectively) at d 20. The birds were gavaged with $10^9$ CFU/bird *C. perfringens* at d 25 and 26. Body weight gain was measured at d 0, 20, 25, and 27. Five birds with representative BW of pen were euthanized at d 27. All graphs depict mean ± SEM.
Figure 2.4  DCA attenuated acute NE-induced BWG lost during d 20-27 in experiment 3.

Birds were raised and infected as in Figure 2.3. Shown is BWG during d 20 to 25 (coccidiosis phage) and d 25 to 27 (NE phase). All graphs depict mean ± SEM. *P < 0.05.
Figure 2.5  DCA attenuates subclinical NE-induced intestinal inflammation in experiment 1.

Birds were raised and infected as in Figure 2.1. Small intestine sample was collected, stained with H&E, and evaluated with histopathological scores. (A) H&E staining showing representative intestinal histology images. (B) Quantification of histological intestinal damage score. All graphs depict mean ± SEM. Scale bar represents 200 μM. *P < 0.05.
Figure 2.6  DCA attenuates acute NE-induced intestinal inflammation in experiment 3.

Birds were raised and infected as in Figure 2.3. Small intestine sample was collected, stained with H&E, and evaluated with histopathological scores. (A) H&E staining showing representative intestinal histology images. (B) Quantification of histological intestinal damage score. All graphs depict mean ± SEM. Scale bar represents 200 μM. *P < 0.05.
Figure 2.7  DCA reduced NE-induced inflammatory gene expression.

Birds were raised and infected as in Figure 2.1. Small intestine sample was collected for RNA extraction. Inflammatory gene expression of $\text{Inf}\gamma$, $\text{Litaf} (\text{Tnf}\alpha)$, and $\text{Il1}\beta$ was quantified with real time PCR and normalized to $\text{Gapdh}$. All graphs depict mean ± SEM. *$P < 0.05$. 


Figure 2.8  DCA reduced NE associated increased *C. perfringens* luminal colonization.

Birds were raised and infected as in Figure 2.1. The intestinal content/digesta was collected for DNA extraction. Luminal *C. perfringens* colonization level quantified by 16 S rDNA real-time PCR. All graphs depict mean ± SEM. *P < 0.05.
Birds were raised and infected as in Figure 2.1. Ileal digesta was collected and the bile acids composition was analyzed using targeted metabolomic analysis (LC-MS/MS). (A) Total bile acid quantification in ileum. (B) Relative percentage of bile acids in ileum content. All graphs depict mean ± SEM. *P < 0.05.
2.7 Reference


Chapter III:

Evaluation of Reconstituting Chicken Microbiota on Broiler Chicken Growth Performance
3.1 Abstract

Industrialization of the agricultural field has fundamentally changed the production practices and the environment within which animals interact. Antimicrobial overuse associated with animal industrialized production is a partial cause of antimicrobial resistance. It is urgent to find antimicrobial free alternatives as growth promoters in the poultry industry, but few effective antimicrobial alternatives are currently available. The objective of this study was to modulate chicken microbiota on the bird growth performance. Mouse specific pathogen free (SPF) stool was cultured on Brain Heart Infusion (BHI) agar under anaerobic or aerobic conditions and collected as SPF anaerobic microbiota (SPF-Anaero) and aerobic microbiota (SPF-Aero). Day-old birds were tagged, weighed and randomly assigned to 9 pens. The birds were orally gavaged with PBS, $10^8$ CFU/bird SPF-Anaero, or $10^8$ CFU/bird SPF-Aero. The bird weight was measured at d 0, 14, and d 28. The broiler chickens were euthanized at d 28. Body weight gain was analyzed using one-way ANOVA followed by $t$-test. Interestingly, birds colonized with SPF-Anaero grew 15% heavier during the starter period of d 0-14 (28 vs. 24 g/day/bird, $P=0.0009$) compared to control birds without microbiota colonization, while birds colonized with SPF-Aero gained 8% more body weight compared to control birds (26 vs. 24 g/day/bird, $P=0.02$). Consistently, SPF-Anaero birds grew faster compared to control birds during d14-28 (67 vs. 60 g/day/bird, $P=0.007$). As a result, SPF-Anaero and SPF-Aero birds grew 15 and 4%, respectively, heavier on accumulative body weight gain compared to control birds (47 vs. 42 g/day/bird, $P=0.0009$; 44 vs. 42 g/day/bird, $P=0.03$, respectively). In conclusion, microbiota as an antimicrobial free alternative is able to improve bird body weight gain. Specific members of the microbiota could be identified, isolated and applied in the poultry industry.
3.2 Introduction

Approaches in agricultural production such as raising poultry have changed for the past 10,000 years, with increasing speed and influence over the recent centuries (Godfray et al., 2010). The speed of agricultural transition to an industrialization system has accelerated and deepened since the 19th century, and industrialized production of food animals began in the poultry sector in the United States in the 1920s (States, 2020). Like wild birds, chicken hens have laid and hatched eggs and raised their chicks in some area of modern countryside and in pre-industrialization era. During the industrialization transition of massive poultry production, eggs laid by hens were hatched in a hatchery and the chicks were raised apart from their parents. In this practice, the loss of microbiota transmission from parents to offspring chickens are apparent and the consequence is being recognized in the era of growing microbiome research.

Poultry meat from chickens or turkeys, is the most consumed animal protein (States, 2020). Antibiotic growth promoters (AGPs) have been widely used in the poultry industry as a growth promoter and disease prevention for decades (Lin, 2014). However, with restricting AGPs by the European Union (EU) in 2006 in association with consumers’ demand and pressure around the world, rearing chickens without AGPs has experienced a variety of challenges (Engster et al., 2002). In addition to the decreasing the overall performance of the birds after withdrawing AGPs (Engster et al., 2002), enteric diseases such as Clostridium perfringens induced necrotic enteritis and others enteric diseases have also emerged (M'Sadeq et al., 2015). Active research has been undertaken for cost effective alternatives to AGPs. Microbial products and individual or groups of bacteria have been reported to be effective, such as short chain fatty acid butyrate and probiotics, at some degree (Bortoluzzi et al., 2017; Lutful Kabir, 2009). The intestine harbors a complex community of trillions of microbes including bacteria, archaea, virus and eukarya. These microbes
called microbiota, and their metabolic activities and products are collectively defined as the microbiome (Sun and Jia, 2018). Improved feed efficiency of chickens is associated with different gut microbiota (Stanley et al., 2013), maybe through extracting nutrients from microbial activities such as short chain fatty acids (acetate, propionate, butyrate, and lactate), enzymes, amino acids, and vitamins B and K (Ewing & Cole, 1994) (Yadav & Jha, 2019). Early colonization of the gut microbiota is important for poultry health and performance (Kers et al., 2018).

Recently, we found that microbial metabolite secondary bile acid deoxycholic acid (DCA) improves broiler chicken body weight gain (Alrubaye et al., 2019) and prevents *Eimeria maxima* and *Clostridium perfringens*-induced acute necrotic enteritis (Wang et al., 2019). In this study, we reconstituted chicken intestinal microbial communities using specific pathogen free (SPF) mouse microbiota. We found that SPF anaerobe and aerobe microbiota improve chicken body weight gain. The results from this study may help in developing new antimicrobial free alternatives.
3.3 Materials and Methods

Chicken feeding and management

All the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas. The experiments were conducted at JKS Poultry Health Laboratory at University of Arkansas at Fayetteville. Day old Cobb 500 male broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR). Chicks were received, neck-tagged and randomly assigned to floor pens in an environmentally controlled house. No medication was added to the feed. Feed and water were supplemented ad libitum and temperature was maintained according to their age, 34°C for the first 5 days and then gradually decreased to 23°C until final day 27. The broiler chicks were fed a corn-soybean meal-based starter diet for the first d 0 to 10 and a grower diet during d 11 to 28. The basal diet was formulated as described before (Alrubaye et al., 2019).

Preparation of the microbiota culture and inoculation of day-old chickens

Mouse specific pathogen free (SPF) microbiota were cultured on Brain Heart Infusion (BHI) agar under anaerobic or aerobic conditions and collected as SPF-Anaero and SPF-Aero, respectively, similar to the previous report (Sun et al., 2018). Day-old birds were then orally gavaged with a single dose of $10^8$ CFU/bird SPF-Anaero, or $10^8$ CFU/bird SPF-Aero microbiota and negative control broiler chickens were gavaged with PBS. The birds were weighed at day 0, 14, and d 28 and were euthanized at d 28.
Statistical Analysis

Data were first analyzed by One-way ANOVA for significant difference and then \textit{t-test} was used for multiple comparison using Prism 7.0 software. Experiments were considered statistically significant if $P$ values were $< 0.05$. 
3.4 Results and discussion

Although industrialization of the poultry industry and the use of antimicrobial growth promoters have greatly improved the production efficiency, issues such as antimicrobial resistance (Caly et al., 2015) and animal welfare (Ben Sassi et al., 2016), urgently need to be addressed. Among various differences resulting from poultry industrialization, the disruption of microbiota transmission from parental sources has been largely overlooked. In this study, we reasoned that broiler chicks from commercial hatcheries would be colonized with environmental (hatchery, transportation vehicles, and grower farms) microbiota. The environmental microbiota will not always be suitable for chicken growth. To test this hypothesis, we reconstituted broiler chicken microbial communities by transplanting mouse SPF microbiota. The selection of mouse microbiota was based on our previous observations that SPF mice are resistant to foodborne pathogen *Campylobacter jejuni* colonization (Sun et al., 2018) while chickens are susceptible (Alrubaye et al., 2019).

All the birds grew healthy and the control birds without microbiota transplantation grew to 0.421 kg/bird at d 14. Birds colonized with SPF-Anaero grew 15% heavier during starter period of d 0-14 (28 vs. 24 g/day/bird, P= 0.0009) compared to control birds without microbiota colonization (Figure 3.1). Birds colonized with SPF-Aero gained 8% more body weight compared to control birds (26 vs. 24 g/day/bird, P= 0.02). Consistently, SPF-Anaero birds grew 12% faster compared to control birds during d14-28 (67 vs. 60 g/day/bird, P= 0.007), while SPF-Aero failed to significantly increase body weight gain (Figure 3.2). As a result, SPF-Anaero and SPF-Aero birds grew 15 and 4 %, respectively, heavier on accumulative body weight gain during d 0-28 (Figure 3.3) compared to control birds (47 vs. 42 g/day/bird, P= 0.0009; 44 vs. 42 g/day/bird, P= 0.03, respectively).
The underlying mechanism of growth promoting capacity of SPF microbiota is unclear. It is possible SPF microbiota improves nutrient generation, absorption and utilization as well as immune response and stress reduction. Interestingly, one of important risk factors affecting overweight or obesity in humans (Okeke et al., 2014) or mice (Turnbaugh et al., 2006) is the influence of the gut microbiome. The microbiota from obese mice has an increased capacity to harvest energy from the diet and this trait is transmissible (Turnbaugh et al., 2006). In our study, it is possible that SPF microbiota increases energy extraction compared to control birds. Future research on nutrient digestibility and absorption should be investigated.

The microbiota is essential for the inducing, training, and reacting of the host immune system to tolerate or against various foreign agents (Belkaid and Hand, 2014). As a result, the host immune system maintains the symbiotic relationship with the highly diverse and evolving microbiota. Gut permeability for bacterial translocation is increased during pregnancy and lactation, and dendritic cells in the milk contribute to neonatal immune imprinting and tolerance (Perez et al., 2007). It would be helpful to investigate whether the microbiota of SPF-Anaero and SPF-Aero modulate chicken immune response.

In conclusion, reconstituting chicken microbiota using mouse SPF-Anaero improves the bird BWG. This finding may be a feasible antimicrobial alternative in poultry production.
3.5 Figures

Figure 3.1  Periodic daily BWG during d 0-14.

Mouse SPF stool was cultured on BHI agar under anaerobic or aerobic condition and collected as SPF anaerobic microbiota (SPF-Anaero) and aerobic microbiota (SPF-Aero). Day-old birds were tagged and placed in six pens (2 pens/group, 16 birds/pen). The birds were orally gavaged with PBS or $10^8$ CFU/bird SPF-Anaero or SPF-Aero. The bird weight was measured at d 0, 14, and 28. Showed are periodic daily BWG during d 0-14. All graphs depict mean ± SEM. *P < 0.05; ***P < 0.001.
Figure 3.2  Periodic body weight gain during d 14-28.

Birds were colonized with SPF-Anaero and SPF-Aero microbiota as in Figure 3.1. Shown are periodic daily BWG during d 14-28. All graphs depict mean ± SEM. **P < 0.01.
Figure 3.3  Accumulative body weight gain during d 0-28.

Birds were colonized with SPF-Anaero and SPF-Aero microbiota as in Figure 3.1. Showed are accumulative daily BWG during d 0-28. All graphs depict mean ± SEM. *P < 0.05; ***P < 0.001.
3.6 Reference


Chapter IV

Conclusions
4.1 Conclusions

The main objectives of this thesis were to investigate selected microbiota and metabolites thereof as an alternative to antibiotics for growth promotion and disease prevention. Four chicken experiments were performed using microbial metabolic product secondary bile acids (DCA), and SPF anaerobe and aerobe microbiota. Notably, DCA in drinking water prevented NE-induced BWG reduction and pathological lesions. This could be through reducing *C. perfringens* colonization and/or restoring the intestinal bile acid pool. On the other hand, reconstituting chicken microbiota using mouse SPF-Anaero microbiota improved chicken growth performance. In the future, specific members of the microbiota could be identified, isolated and applied in the poultry industry. In conclusion, these findings may offer an alternative strategy to use microbiota and its metabolite for growth promotion and NE prevention.
Chapter V
Appendix

To:    Xiaolan Sun
FR:    Craig Coon
Date:  December 14th, 2016
Subject: IACUC Approval
Expiration Date: November 17th, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 17036: Necrotic enteritis and Clostridium perfringens pathogenesis in chicken.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond November 17th, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study, Xiaolan Sun and Hong Wang. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem